REMARKS

Claims 15-35 are currently pending in the present application. Table 1 on page 5 of the instant specification has been amended to indicate the sequences of the antisense oligonucleotides as disclosed in the application. Support for these amendments can be found in the specification, for example at page 4, lines 4-7 and lines 29-30; page 16, lines 2-7; page 23, lines 13-15; and Figure 7. The specification has additionally been amended to insert the appropriate SEQ ID NO. identifiers with the respective sequence listings. Further, a new sequence listing is submitted herewith as required under 37 CFR 1.821(g). Additionally, applicants submit herewith substitute specification pages with the corrections already made for the Examiner's convenience.

The claims have additionally been amended in the expectation that the amendments will place this application in condition for allowance. The amendments do not introduce new matter within the meaning of 35 U.S.C. §132. Accordingly, entry of the amendments is respectfully requested.

The presently pending claims relate to compositions containing an antisense deoxyoligonucleotide having a sequence according SEQ ID NO: 1 (Oligo 83) or SEQ ID NO: 2 (Oligo 86) and

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a pharmaceutically acceptable carrier or diluent in combination with an antiproliferative drug, an anticancer agent or a thymidylate synthase (TS) inhibitor. The claims also relate to methods of using the compositions and the combinations to treat cancer, to inhibit tumor cell growth or proliferation, to sensitize mammalian tumor cells to anticancer agents or to inhibit TS expression in mammalian cells.

1. Rejection of Claims 15-35 under 35 U.S.C. § 112, 2nd paragraph

The Official Action states that claims 15-35 are rejected under 35 U.S.C. 112, second paragraph, as failing to set forth the subject matter which applicant regards as the invention. Applicants respectfully traverse this rejection. In particular, the Examiner asserts at page 3, lines 2-5 of the Official Action that, "contrary to the Applicant's assertions, the sequences according to SEQ ID NO: 1-2 represent target regions of TS mRNA, and do not represent sequences that would hybridize 'antisense' or a reverse compliment of the targeted regions of TS mRNA."

Applicants respectfully traverse this rejection. Regarding the §112, second paragraph rejection, caselaw has defined two requirements under the statute: (1) whether the applicant has stated the invention as something elsewhere in the application which would not fall under the scope of the claims; and (2)

whether the claims would be communicated with a <u>reasonable</u> degree of particularity and distinctness to a person skilled in the art in light of the content of the disclosure and the teachings of the prior art. MPEP §2171, §2173, and §2173.02.

Contrary to the Examiner's assertions, applicants respectfully submit that one skilled in the art would understand that the targeted mRNA of the presently claimed invention is sense to the cDNA indicated in Figure 7. Moreover, the skilled technician would understand that the presently pending claims pertain to pharmaceutical compositions containing, combinations of, and methods of using oligonucleotides complementary (i.e., antisense) to the delineated regions of the mRNA as represented by the cDNA in Figure 7.

In particular, the instant specification states at page 4, lines 4-7 that, "An antisense oligonucleotide is an oligonucleotide which is designed to hybridize to a specific region of a targeted nucleic acid sequence. The targeted nucleic acid is the TS gene or mRNA transcribed from the TS gene. Preferably the targeted nucleic acid is the mRNA encoding thymidylate synthase."

Further, throughout the instant specification are presented numerous provisions of support for applicants' assertion that

the presently claimed invention comprises antisense

oligonucleotides complementary to the TS cDNA/mRNA of Figure 7.

For example, the instant specification references the GenBank

sequence accession no. X02308. As evidenced by a printout for

this accession number, a courtesy copy of which is attached for

the Examiner's convenience, this entry in GenBank is defined as

"Human mRNA for thymidylate synthase". Further, the identical

sequence as that provided in GenBank is sourced from Takeishi et

al., as referenced in the instant specification at page 27, line

26, and disclosed in the application, in part in Figure 7. The

Examiner is reminded that a reference in a patent specification

to a deposit in a public repository (such as the GenBank

sequence described above) is sufficient to constitute an

adequate description of the deposited material to satisfy the

requirements of 35 U.S.C. 112. See Enzo Biochem, Inc. v. Gen-

Probe, Inc., 63 U.S.P.Q.2d 1609 (Fed. Cir. 2002).

Similarly, the instant specification describes the

antisense oligonucleotides of the invention "using the sequence

numbering described for human thymidylate synthase mRNA by

Takeishi et al., 1985" (page 3, lines 24-25 and page 4, lines 1-

3). For example, page 16, lines 2-4 states that Oligo 86 (SEQ

ID NO: 2) is "complementary to TS mRNA from base pair positions

1035 to 1054 (GenBank accession no. X02308; Takeishi *et al.*, 1985), which surround the TS mRNA translation stop site (UAG at bases 1045 to 1047)." Accordingly, the repeated references in the instant specification to the antisense oligonucleotides of the invention as "targeted" to or "complementary" to the sequence of GenBank sequence accession no. X02308 supports applicants' assertion that the presently claimed compositions, combinations, and methods comprise antisense oligonucleotides complementary to the TS cDNA/mRNA of Figure 7.

Additionally, page 23, lines 10-14 of the instant specification describes the phosphorothiorated oligonucleotide of SEQ ID NO: 1 (termed ODN 83) as "complementary to TS mRNA, starting from a position 136 base downstream of the translation stop site (5'-GCCAGTGGCAACATCCTTAA-3')." Accordingly, the instant specification provides explicit support for the sequences of the antisense oligonucleotides comprised within the presently claimed compositions, combination products, and methods of use. Likewise, the instant specification also describes examples employing oligonucleotides antisense to regions of the TS mRNA in combination with an anticancer agent, Tomudex, for example, at page 21, lines 17-19.

Applicants have accordingly amended Table 1 on Page 5 of

the instant specification and provided a replacement sequence listing indicating the sequences of the antisense oligonucleotides disclosed in the application, as described above. Further support for these amendments can be found in the specification, for example at, lines 4-7 of page 16.

Applicants submit, then, that the presently claimed invention does in fact comprise compositions, combinations products, and methods of using antisense oligonucleotides complementary to the TS cDNA/mRNA of Figure 7, contrary to the Examiner's assertion. The instant specification provides numerous instances of support for this assertion.

Accordingly, applicants respectfully request the Examiner to reconsider and withdraw the rejection of pending claims 15-35.

2. Rejection of Claims 15-35 under 35 U.S.C. § 112, 1st paragraph

The Official Action states that claims 15-35 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. In particular, the Examiner asserts that the "specification fails to provide sufficient guidance to the

skilled artisan on the parameters for practicing a method of nucleic acid therapy in an individual *in vivo* comprising administration of a complex comprising TS antisense oligonucleotides for the breath of the claimed invention." Further, the Examiner maintains that applicants need to provide evidence that the presently claimed antisense pharmaceutical compositions and methods for treatment produce a therapeutic effect *in vivo* as well as guidance on how to produce this effect without undue experimentation.

Applicants respectfully traverse this rejection. In order to make an enablement rejection, the Examiner has the initial burden to establish a *reasonable* basis to question the enablement provided for the claimed invention. *In re Wright*, 27 USPQ2d 1510 (Fed. Cir. 1993). The test under 35 U.S.C. § 112, first paragraph, for determining compliance with the enablement requirement is whether one skilled in the art could make or use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *United States v. Telectronics, Inc.*, 8 USPQ2d 1217 (Fed. Cir. 1988).

Initially, applicants traverse the Examiner's assertion that the antisense deoxyoligonucleotides sequences recited in

the presently claimed compositions, combination products, and methods of use are not antisense sequences. Instead, as applicants have shown in section 1 above, the arguments of which are herein incorporated by reference in their entirety, these sequences are in fact antisense sequences.

Further, in reply to the Examiner's requirement for *in vivo* evidence, applicants remind the Examiner that, as stated in the Response to Official Action of March 5, 2002, "*in vivo* data is not required under 35 USC §112, first or second paragraph. A specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as if in compliance with the enabling requirement of the first paragraph of §112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support. *In re Marzocchi*, 169 USPQ 367, 369 (CCPA 1971); MPEP 2107.01."

However, for the Examiner's information, applicants further enclose with this response a courtesy copy for the Examiner's review of the following journal article disclosing an *in vivo* study showing that systemic treatment with TS antisense ODN 83

significantly inhibited (HT29 human colon carcinoma cell) tumor growth in mice published by the present applicants. The reference is as follows, Berg, R.W., et al., "Tumor Growth Inhibition *in Vivo* an G2/M Cell Cycle Arrest Induced by Antisense Oligodeoxynucleotide Targeting Thymidylate Synthase", (2001) *J. of Pharmacology and Experimental Therapeutics*, 298 (2), 477-484.

The Examiner's attention is respectfully directed to the experimental protocol ("Animal Studies") portion of this journal article, which indicates that tumors were first established by subcutaneous injection of cancer cells in mice and Oligo 83 subsequently injected intraperitoneally into the mice. The results of the animal studies indicate that Oligo 83 was able to retard tumor growth.

Accordingly, in view of applicants own additional published *in vivo* data demonstrating the ability of the presently claimed antisense deoxyoligonucleotides to effectively retard tumor growth, applicants assert that one skilled in the art would have a reasonable expectation, based on the teachings in the application, that the invention would work as claimed without the application of undue experimentation. The Examiner is reminded that a specification must be viewed as enabling unless

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the Examiner is able to provide evidence to the contrary (*In re Wright*). In view of this additional *in vivo* data, the Examiner has no basis for asserting that the present specification is non-enabling unless she can provide <u>specific evidence</u> to the contrary.

Accordingly, applicants respectfully request the Examiner to reconsider and withdraw the rejection of pending claims 15-35.

CONCLUSION

Based upon the foregoing amendments and remarks, the presently claimed subject matter is believed to be enabled, novel, and patentably distinguishable over the prior art of record. The Examiner is therefore respectfully requested to reconsider and withdraw the outstanding rejections and allow all pending claims 15-35 presented herein for reconsideration. Favorable action with an early allowance of the pending claims is earnestly solicited.

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The Examiner is invited to telephone the undersigned attorney if she has any questions or comments.

Respectfully submitted,

NATH & ASSOCIATES PLLC
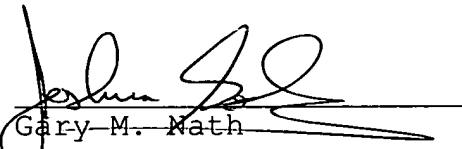
Date: _July 16, 2003_
NATH & ASSOCIATES PLLC
1030 15th Street, N.W., 6th Floor
Washington, D.C. 20005-1503
Tel: (202) 775-8383
Fax: (202) 775-8396
GMN:TLJ:JBG:\RFR.doc

Gary M. Nath
Reg. No. 26,965
Joshua B. Goldberg
Reg. No. 44,126
Customer No. 20529

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Table 1:

ANTISENSE OLIGONUCLEOTIDE	SEQUENCE
OLIGO 83 (SEQ ID NO: 1)	GCCAGTGGCAACATCCTTAA
OLIGO 86 (SEQ ID NO: 2)	AAGCACCCTAAACAGCCATT
OLIGO 90 (SEQ ID NO: 3)	GCAGCTCCGAGCCGGCCACA
OLIGO 91 (SEQ ID NO: 4)	GCCGGCCACAGGCATGGCGC
OLIGO 92 (SEQ ID NO: 4)	GCCGGCCACAGGCATGGCGC
OLIGO 93 (SEQ ID NO: 6)	GGCATGGCGCGGCGGGCGGG
OLIGO 81 (SEQ ID NO: 10)	CTCAGCTCCCTCAGATTTG
ODN 32 (SEQ ID NO: 7)	ATGCGCCAACGGTTCCTAAA
PAS/TSS (SEQ ID NO: 8)	UGUGGCCGGC UCGGAGCUGC CGCGCCGGCC
PAS/EXON1,2 (SEQ ID NO: 9)	GCUACAGCCU GAGAGAUGAA UUCCCUCUGC

It will be appreciated that the invention is not restricted merely to those specific antisense oligonucleotides which are disclosed in Table 1 above but encompasses

5   oligonucleotides of from about 8 to about 50 nucleotides in length which selectively inhibit or selectively enhance thymidylate synthase production and which are selected from those regions of the TS gene which are described hereinbefore.

Hybridisation of an antisense oligonucleotide to its target nucleic acid sequence is mediated by the formation of hydrogen bonds between complementary bases on each nucleic

10 acid strand. Hybridisation may occur between nucleic acid strands which have varying degrees of complementarity, depending on the hybridisation conditions employed. The term "specifically hybridisable" is used to describe an oligonucleotide which has a sufficient degree of complementarity to ensure stable, specific binding to its target sequence, whilst avoiding non-specific binding to non-target sequences.

15 Antisense oligonucleotides may be designed to hybridise to any region within the thymidylate synthase mRNA molecule, including the coding region, the 5'untranslated region, the 3'untranslated region, the 5'cap region, introns and intron/exon splice junctions.

Hybridisation of the antisense oligonucleotide to thymidylate synthase mRNA may affect any aspect of mRNA function, for example mRNA translocation, mRNA splicing,

20 mRNA translation, or the feedback inhibition mechanism regulated by the binding of

*: Significantly higher than control (p<0.05, one way analysis of variants).

**: Significantly lower than control (p<0.05, one way analysis of variants).

Figure 2 shows that HeLa cell growth is inhibited by transfection with antisense TS oligo 86 (targeted to the translation stop site), but is enhanced after transfection with antisense TS oligo 91 (targeted to the translation start site).

HeLa cells were transfected with 0.05 or 0.10 μM antisense TS oligonucleotides in Lipofectin (10 μg/ml) for 4 hours as described. Note that oligo concentrations are considerably lower than those used for MCF-7 cells. The efficiency of Lipofectin-mediated DNA transfection of HeLa cells is greater than for MCF-7 cells. Lipofectin was removed, the cells were trypsinised, and 25,000 viable cells per flask were plated in tissue culture flasks. Cell numbers were measured by Coulter counter in triplicate flasks after 4, 7 and 8 days of growth. Control cells were treated with Lipofectin without oligonucleotides. Cell growth is expressed as a percentage of growth of control cells.

*: Significantly higher than control (p<0.05, Student's t-test).

**: Significantly lower than control (Student's t-test).

Figure 3 shows that HeLa cell growth is inhibited by transfection with antisense TS oligo 83 (targeted to a 3' untranslated seqeunce downstream of the translation stop site), but it is not affected by transfection with antisense TS oligo 81 (targeted to a sequence in the 3' untranslated region of TS mRNA). The experimental protocol was as described in the legend to Figure 2.

Figure 4 shows that transient transfection of HeLa cells with oligo 86 (targeted to the TS translation stop site) enhances sensitivity to Tomudex and that oligo 91 (targeted to the TS translation start site) reduces sensitivity to Tomudex.

HeLa cells were transfected with 0.05 and 0.10 μM antisense TS oligonucleotides and plated in flasks at low density, as described for Figure 2. Tomudex (0-8 nM) was added (triplicate flasks for each Tomudex concentration) and the cells allowed to proliferate for 7 days. Cell numbers were measured by Coulter counting at that time. Survival is plotted as a percentage of growth in cells transfected with oligonucleotide, but untreated with Tomudex. Therefore, these data reveal inhibition or enhancement of Tomudex killing independent of

growth inhibition or enhancement induced by oligonucleotides in the absence of Tomudex. The mean of three values is plotted. Error bars were smaller than the size of the symbol in each case.

*: Significantly different from control (p<0.05, Student's t-test).

Figure 5 shows that transient transfection of HeLa cells with oligo 83 (targeted to a sequence in the 3' untranslated region of TS mRNA) enhances sensitivity to Tomudex whereas oligo 81 (targeted to a 3' seqeunce downstream of that targeted by oligo 83) has no effect on Tomudex sensitivity.

HeLa cells were transfected with 0.10 μM antisense TS oligonucleotides and plated in flasks at low density, as described for Figure 2. Tomudex (0-10 nM) was added (triplicate flasks for each Tomudex concentration) and the cells allowed to proliferate for 4 days. Cell numbers were measured by Coulter counting at that time. Survival is plotted as a percentage of growth in cells transfected with oligonucleotide, but untreated with Tomudex. Therefore, these data reveal enhancement of Tomudex killing independent of growth inhibition induced by oligonucleotides in the absence of Tomudex. The mean of three values is plotted. Error bars were smaller than the size of the symbol in each case.

*: Significantly different from control (p<0.05, Student's t-test).

Figure 6 shows that antisense TS oligo 91, but not oligo 86, stimulates TS gene transcription in human HeLa cells.

The same HeLa cells for which data are presented in Figure 2 were assessed for run-on transcription of TS, glyceraldehyde phosphate dehydrogenase (GAPDH), and 18S rRNA genes. Briefly, cells were transfected with 0.05 and 0.10 μM antisense TS oligonucleotides in Lipofectin (10 μg/ml), or with Lipofectin alone (LFA control) for 4 hours as described. Lipofectin was removed and cells were trypsinised and replated in tissue culture flasks. Four days after transfection, nuclei were isolated from approximately 5×10^6 cells for each treatment and initiated TS, GAPDH, and 18S rRNA transcripts allowed to incorporate [^{32}P]-CTP for 30 minutes. Alcohol-precipitable radiolabeled RNA was hybridised for 48 hours to unlabeled TS, GAPDH, and 18S rRNA cDNA immobilised in triplicate dots on nylon membrane as described. Relative transcription rate is presented as:

Bases in bold-face (below) form part of restriction endonuclease sites, and are not sense or antisense TS sequences. Numbering indicates the distance from the beginning of the transcription start site.

5   *TS cDNA nucleotides 111 to 140*

sense TS (JK-5):   **CTAGA**TGTGGCCGGCTCGGAGCTGCCGCGCCGGCCA (SEQ ID NO: 11)
antisense TS (JK-2):   **AGCTT**GGCCGGCGCGGCAGCTCCGAGCCGGCCACAT (SEQ ID NO: 12)

TS cDNA nucleotides 296 to 325

10   *sense TS (JK-3):*   **CTAGA**GCTACAGCCTGAGAGATGAATTCCCTCTGCA (SEQ ID NO: 13)
antisense TS (JK-4):   **AGCTT**GCAGAGGGAATTCATCTCTCAGGCTGTAGCT (SEQ ID NO: 14)

<u>*MT-2 oligonucleotide probes (Karin and Richards, 1982):*</u>

Sense and antisense oligonucleotide sequences did not have non-complementary

15 sequences added to the 5' and 3' ends. Numbering indicates the distance from the translation start site.

MT cDNA nucleotides -14 to 6

sense MT: CTCTTCAGCACGCCATGGAT (SEQ ID NO: 15)

20   *MT cDNA nucleotides 204 to 223*

antisense MT: AGGGTCTACCTTTCTTGCGC (SEQ ID NO: 16)

Example 1.2: Antisense oligodeoxynucleotide targeting regions at or near the translation stop site at the 3' end of the TS gene as a method to inhibit growth of human tumour cells

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a) A 20-mer antisense oligodeoxynucleotide (oligo 86) targeted to the translation stop site at the 3' end of the thymidylate synthase mRNA is growth inhibitory (cytostatic) in a human breast cancer cell line (MCF-7 cells). Antisense oligonucleotides of the same length (oligos 90 and 92), targeted to regions at or near the translation start site at the 5' end of the

30 TS mRNA, are not cytostatic **(Figure 1).**

hr. The enzyme was inactivated at 95°C for 5 min. The resulting cDNAs (in a volume of 2.5 μl) were amplified in a polymerase chain reaction (PCR) using 1.25 U of *Taq* DNA polymerase in 50 μl of 20 mM Tris-HCl pH 8.4), 50 mM KCl, 0.2 mM mixed dNTPs, 2 mM MgCl$_2$, and 50 pmol of primers specific for TS and GAPDH cDNAs. TS and GAPDH cDNAs were amplified together in the same reaction tube to allow the level of housekeeping GAPDH cDNA to be used to determine the relative level of TS mRNA. Twenty-four to 27 cycles of PCR amplification (94°C 45 s, 55°C 30 s, 72°C 90 s) produced fragments of 208 bp and 752 bp using primer sets for TS (forward 5'CACACTTTGGGAGATGCACA3'(SEQ ID NO: 17); reverse 5'CTTTGAAAGCACCCTAAACAOCCAT3'(SEQ ID NO: 18)) and GAPDH (forward 5'TATTGGGCGCCTGGTCACCA3' (SEQ ID NO: 19); reverse 5'CCACCTTCTTGATGTCATCA3') (SEQ ID NO: 20),

respectively. PCR products were separated on a 1.2% agarose gel, and transferred to Hybond nylon membrane (Amersham, Canada, Ltd., Oakville, Ontario, Canada) by Southern blotting. Blots were hybridised to [α-^{32}P]dCTP random primer-labeled probe (pcHTS-1, a generous gift from Dr. K. Takeishi, University of Shizuoka, Shizuoka, Japan; or a cDNA insert recognising glyceraldehyde-3-phosphate dehydrogenase [GAPDH]). Hybridisation signals were quantified using a PhosphorImager and ImageQuant (Molecular Dynamics, Sunnyvale, California, USA).

TS binding assay:

Cellular content of TS was assayed by binding of [6-^{3}H]5-FdUMP. This method was demonstrated to label total TS unless the cells were pretreated with 5-FU or 5-FUdR. The assay was performed using cells that were treated with antisense ODN 83 or the scrambled control ODN 32. Briefly, cells were harvested by scraping into PBS and resuspending the subsequent pellet in 100 mM KH$_2$PO$_4$ (pH 7.4). Cells were disrupted by freezing and thawing, followed by sonication. The total protein concentration was determined using Coomassie staining (BioRad reagent) (MI) in order to express results as pmol 5-FdUMP bound per mg total protein. 5-FdUMP binding was assessed in paired lysates from cells transfected with ODN 83 or ODN 32, in separate incubation reactions carried out on different days; however, pairs were always assessed together under the same reaction conditions. On each occasion, the incubation vessel contained 50 μg of total protein, 75 μM methylene-FH$_4$, 100 mM mercaptoethanol, 50 mM KH$_2$PO$_4$ (pH 7.4), and 15 nM [6-^{3}H]5-FdUMP in a final

NCBI Nucleotide

PubMed   Nucleotide   Protein   Genome   Structure   PMC   Taxonomy   OMIM   Book

Search Nucleotide ▨ for [   ]   Go Clear

Limits   Preview/Index   History   Clipboard   Details

Display GenBank ▨ Show: 20 ▨ Send to File ▨   Get Subsequence

☐ 1: X02308. Human mRNA for th...[gi:37478]   Links

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LOCUS       HSTSYN1                 1536 bp    mRNA    linear   PRI 12-SEP-1993
DEFINITION  Human mRNA for thymidylate synthase (EC 2.1.1.45).
ACCESSION   X02308
VERSION     X02308.1  GI:37478
KEYWORDS    inverted repeat; synthetase; tandem repeat.
SOURCE      Homo sapiens (human)
  ORGANISM  Homo sapiens
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            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (bases 1 to 1536)
  AUTHORS   Takeishi,K., Kaneda,S., Ayusawa,D., Shimizu,K., Gotoh,O. and
            Seno,T.
  TITLE     Nucleotide sequence of a functional cDNA for human thymidylate
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  JOURNAL   Nucleic Acids Res. 13 (6), 2035-2043 (1985)
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Jun 19 2003 12:37:45

Tumor Growth Inhibition in Vivo and G₂/M Cell Cycle Arrest Induced by Antisense Oligodeoxynucleotide Targeting Thymidylate Synthase

RANDAL W. BERG, MELANIE WERNER, PETER J. FERGUSON, CARL POSTENKA, MARK VINCENT, D. JAMES KOROPATNICK, and ELKE BEHREND

Cancer Research Laboratories, The London Regional Cancer Centre, London, Ontario, Canada (R.W.B., M.W., P.J.F., C.P., E.B., M.V., D.J.K.); and Departments of Oncology (M.V., D.J.K.), Microbiology and Immunology, Pathology, and Pharmacology and Toxicology (D.J.K.), The University of Western Ontario, London, Ontario, Canada

ABSTRACT

Chemotherapeutic agents targeting thymidylate synthase (TS) are effective against human tumors. Efficacy is limited by drug resistance, often mediated by TS overexpression. Treatment of HeLa cells in vitro with an antisense oligodeoxynucleotide (ODN 83) targeting human TS mRNA reduces TS mRNA and protein levels, inhibits cell proliferation, and sensitizes cells to TS-targeting drugs (Ferguson et al., 1999). The present study investigates the mechanism by which ODN 83 inhibits cell proliferation and examines its antitumor efficacy in vivo. ODN 83 treatment did not induce apoptosis in HeLa cells in vitro but caused accumulation of cells at G₂/M. In contrast, TS-targeting chemotherapeutics arrest at G₁ or S. Antisense down-regulation reduced TS mRNA levels in human colon cancer (HT29) cells by 40% in vitro, resulted in G₂/M arrest, and reduced proliferation without enhanced cell death. Growth of HT29 tumors in immunocompromised mice was significantly inhibited when antisense ODN 83 treatment began promptly after tumor implantation and was accompanied by a 40% reduction in TS protein levels. Growth of tumors allowed to reach 400 mm³ prior to ODN administration was unaffected by antisense ODN 83. Radiolabeled ODNs were localized to the tumor periphery but evenly distributed in normal tissue. Thus, down-regulation of TS mRNA and protein by antisense ODN treatment exerts a novel G₂/M cell cycle block without increasing cell death and inhibits HT29 tumor cell growth in vivo. Antisense ODN 83 may be an effective therapy for colon carcinoma, alone or in combination with TS-targeting cytotoxic drugs.

Thymidylate synthase (TS) is an essential enzyme for de novo synthesis of thymidylate and is required for DNA replication. It plays an important role in cell proliferation and is an important target for antitumor therapies designed to reduce the proliferative capacity of cancer cells (Danenberg et al., 1999). Chemotherapeutic drugs that target TS include 5-fluorouracil (5-FU) and raltitrexed (Tomudex) (Papamichael, 1999). Although these drugs have had clinical success, resistance to TS inhibitors often develops both in vitro and in vivo (Gorlick and Bertino, 1999). Drug resistance can be mediated by a variety of mechanisms, including increased TS levels resulting from increased TS transcription (Shibata et al., 1998) and translation (Keyomarsi et al., 1993), and is a major obstacle to clinical efficacy.

A growing number of cellular components are being targeted for therapeutic down-regulation by use of antisense

oligodeoxynucleotides (ODNs). Antisense ODNs specifically decrease protein expression by base-pairing with a specific mRNA to block translation and/or induce cleavage by ribonuclease H (Stein and Cheng, 1993). Antitumor effects have been observed in tumor-bearing animals in response to antisense ODNs against targets as varied as the proto-oncogenes c-myb (Del Bufalo et al., 1996) and c-myc (Leonetti et al., 1996), matrilysin (matrix metalloproteinase-7) (Hasegawa et al., 1998), the antiapoptotic protein Bcl-2 (Miayake et al., 2000), and protein kinase Cα (Dean et al., 1996). Phosphorothioate ODNs administered to mice were found at highest concentrations in the liver and kidney and were stable in most tissues for more than 48 h (Agrawal et al., 1991; Saijo et al., 1994). Although the accumulation and stability of ODNs in tumor tissue in vivo were demonstrated in these two studies, their distribution within a tumor mass has not been described.

We have previously shown that treatment of HeLa cells in

ABBREVIATIONS: TS, thymidylate synthase; ODN, oligodeoxynucleotide; 5-FU, 5-fluorouracil; 5-FUdR, 5-fluorodeoxyuridine; 5-FdUMP, 5-fluorodeoxyuridine monophosphate; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

viuo with an antisense ODN targeting TS decreases TS mRNA and protein levels, inhibits cell proliferation, and sensitizes HeLa cells to 5-FU, 5-fluorodeoxyuridine (5-FUdR), and raltitrexed (Ferguson et al., 1999). Antisense ODNs against TS might be expected to block DNA synthesis and arrest cells in G_1 or early S phase, similar to the action of 5-FU (Inaba and Mitsuhashi, 1994) and raltitrexed (Matsui et al., 1996; Yin et al., 1999). However, TS protein binds to a variety of mRNA molecules, including those encoding p53 (Chu et al., 1996; Ju et al., 1999), c-*myc* (Chu et al., 1995), and TS itself (Chu et al., 1991, 1994). This has raised the possibility that post-transcriptional regulation of mRNA metabolism by TS protein might control not only TS protein production but also that of cell cycle regulatory proteins. Treatment of cells with TS antisense ODNs to decrease both TS mRNA and protein levels may lead to cell cycle perturbations that are not predicted from experiments using protein-targeting drugs that act after mRNA translation.

To examine the cytotoxic and cytostatic effects of targeting TS with antisense ODNs, we have measured apoptosis in HeLa cells and analyzed the cell cycle distribution of HeLa and HT29 carcinoma cells treated in vitro with TS antisense ODN 83. In addition, to test the hypothesis that antisense down-regulation of TS would be an effective antitumor strategy in vivo, we have assessed the antitumor activity of TS antisense ODN 83 on the growth of HT29 tumor explants in immunocompromised mice. We report that the TS antisense ODN 83 has novel and potentially therapeutically exploitable effects on human tumor cell cycle and viability in vitro and in vivo.

Materials and Methods

Cell Culture. HeLa and HT29 cells (from the American Type Culture Collection, Manassas, VA) were grown in Dulbecco's modified Eagle's medium and RPMI1640 (respectively) containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2. All tissue culture reagents were from Invitrogen Canada (Burlington, ON, Canada).

Oligodeoxynucleotides and Transfection. The TS antisense ODN 83 (5 -GCCAGTGGCAACATCCTTAA-3) is complementary to the sequence 136 to 155 base pairs downstream of the translation stop site in the 3 untranslated region of human TS mRNA. There are no other known mRNAs (including mouse TS) with sequences complementary to ODN 83. The control scrambled ODN 32 (5 ATGCGCCAACGGTTCCTAAA-3) has the same base composition in random order. For in vivo studies, ODNs with phosphorothioate linkages between the six nucleotides at both the 5 - and 3 -ends were purchased from BioCorp Inc. (Montreal, QC, Canada). For in vitro studies, fully phosphorothioated ODNs with 2 -methoxy-ethoxy modification on the six nucleotides at both the 5 - and 3 -ends were generously provided by Dr. N. Dean (ISIS Pharmaceuticals, Carlsbad, CA).

HeLa and HT29 cells were transfected with ODNs using LipofectAMINE (Invitrogen Canada), as described (Ferguson et al., 1999), or with LipofectAMINE 2000 (Invitrogen Canada) as described below. For HT29 cells, preliminary experiments indicated that LipofectAMINE 2000 was the superior lipid formulation and was effective at 1 to 5 μg/ml, although nonspecific toxicity was apparent at the higher doses. Concentrations of ODN 83 from 50 to 200 nM were found to be effective at inhibiting HT29 cell proliferation, with improved activity at the higher concentrations. For proliferation assays, HT29 cells were plated at 2 10^5 cells per 25-cm^2 flask in 2 ml of medium. On the following day, a 6   transfection mix was prepared containing 600 nM ODN and 6 μg/ml LipofectAMINE

2000 in serum-free medium. After incubation for 15 min at room temperature, 5 volumes of medium with 10% serum were added, and the medium on the cells was replaced with the 1   transfection mix. For flow cytometry samples and RNA preparation, cells were plated at 1 to 2 10^6 cells per 75-cm^2 flask in 5 ml of medium. The next day, 1 ml of a DNA/lipid mixture in serum-free medium was added directly to each 75-cm^2 flask to yield final concentrations of 200 nM ODN and 2 μg/ml lipid.

Animal Studies. Female nude mice (N:NIH-*bg-nu-xid*), purchased from Charles River Laboratories (St. Constant, QC, Canada), were housed and cared for according to standards of the Canadian Council for Animal Care and were used under a protocol approved by the University of Western Ontario Council on Animal Care. TS-inhibitory cytotoxic drugs are a mainstay in the treatment of colon cancer (Papamichael, 1999), and preliminary studies indicated that human HT29 colon carcinoma cells grow well in these immunocompromised mice (E. Behrend, unpublished observations). To assess the effect of ODN on tumor growth, 4- to 6-week-old mice were injected subcutaneously in the right flank with 5 10^6 HT29 cells on day 0. Every 2nd day beginning on day 1, mice were injected intraperitoneally with ODNs dissolved in 150 mM NaCl. Tumors were measured in two perpendicular dimensions with a caliper every 4 days, and tumor volume was calculated using the formula volume length width^2 π/6. To measure their effect on larger, established tumors, ODNs were administered to mice when the tumors achieved a size of 400 mm^3

Oligodeoxynucleotide Labeling and Distribution Analysis. ODNs (2 μg) were end-labeled to a specific activity of 2 10^8 cpm/μ with [γ^{32}P]adenosine triphosphate (specific activity, 7000 Ci/mmol, ICN Pharmaceuticals, Inc., Costa Mesa, CA) using 20 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) for 10 min at 37°C. Unincorporated radionucleotide was removed using a Sephadex G50 NICK column (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Labeled ODN (200 ng) was mixed with 0.5 mg of unlabeled ODN for each injection. Mice that had been treated with ODN every 2nd day beginning the day after tumor cell implantation, and who were bearing HT29 tumors approximately 800 mm^3 in volume, were injected intraperitoneally two times 4 days apart with the labeled ODN and then sacrificed 2 days later. Various tissues were fixed in neutral-buffered formalin and embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin. PhosphorImage screens were exposed to tissue sections for 3 days and radioactive decay images captured on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Thymidylate Synthase Quantitation. A [6^{3}H]5-FdUMP binding assay (Spears and Gustavsson, 1988) was used to quantitate TS in tumor samples, as described (Ferguson et al., 1999). Tumors were obtained from mice treated with ODN every second day for 28 days, beginning on day 1 after tumor cell implantation. Cell lysates from frozen tumors were prepared by homogenization in 100 mM potassium phosphate, pH 7.4, followed by one cycle of freezing, thawing, and sonication. Supernatants were obtained following centrifugation at 6500 for 30 min at 4°C. Protein concentrations were determined using a protein assay kit (Bio-Rad, Hercules, CA). Aliquots containing 100 μg of total protein were incubated with 75 μM methylene tetrahydrofolate, 100 mM 2-mercaptoethanol, and 15 nM [6-^{3}H]5-FdUMP (specific activity, 18.6 Ci/mmol, Moravek Biochemicals, Brea, CA), in 50 mM potassium phosphate (pH 7.4) for 30 min at 37°C, and the reaction was stopped with 1 ml of albumin-coated, acidified charcoal for 10 min at room temperature. The slurry was centrifuged two times at 5000 for 30 min at 22°C to remove particulate matter, and 400-μl aliquots of the cleared supernatant were assayed by scintillation counting. [6^{3}H]5-FdUMP bound to TS, and therefore unavailable for precipitation with charcoal, was quantitated.

Apoptosis Assay. HeLa cells were treated with 4 μg/ml LipofectAMINE and 50 nM ODN for 6 h, trypsinized, and plated onto coverslips in 12-well plates at 1 10^4 cells/well in culture medium.

Because ODN 83 effectively suppresses, within 24 h, the normal increase in HeLa cell number resulting from proliferation (Ferguson et al., 1999), an earlier time-point was chosen to assess apoptosis. After 15 h, cells were air dried and fixed in 4% paraformaldehyde for 30 min, washed with phosphate-buffered saline, permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate for 2 min at 4°C, and washed with phosphate-buffered saline. Apoptotic cells were detected using the In Situ Cell Death detection kit (Roche Diagnostics, Laval, QC, Canada) and Fast Red (Sigma, St. Louis, MO) as the chromogenic substrate. More than 500 cells were scored as either apoptotic (stained red) or nonapoptotic for each condition.

Flow Cytometry. Cells were collected at 24, 36, 48, and 72 h after ODN treatment, washed with phosphate-buffered saline, fixed in 75% ethanol for 15 min at room temperature, and washed again. Cells treated with 1 μM raltitrexed or 0.1 mM 5-FU for 2 to 4 h, then washed and cultured for 2 days in drug-free medium, were similarly collected. The cells were stained with propidium iodide [0.02 mg/ml in phosphate-buffered saline with 0.1% (v/v) Triton X-100 and 0.2 mg/ml deoxyribonuclease-free ribonuclease A] and analyzed on a Beckman Coulter XL-MCL flow cytometer. At least 10,000 single cells were analyzed for each condition. Analysis regions were set manually to determine the proportion of cells in G_0/G_1, S, and G_2/M cell cycle phases. Alternatively, MultiCycle (version 3.0) software (Phoenix Flow Systems, San Diego, CA) was used to analyze the cell cycle distribution.

Quantitation of TS mRNA by RT-PCR. Total RNA was isolated from HT29 cells treated with ODNs using TRIzol (Invitrogen Canada), and 2 μg of RNA reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen Canada). Two percent of the cDNA produced was used as template for PCR with the primers GAP-for (5 -TATTGGGCGCCTGGTCACCA-3) and GAP-rev (5 -CCACCTTCTTGATGTCATCA-3) for GAPDH, or TS-for (5 TTTTGGAGGAGTTGCTGTGG-3) and TS-rev (5 -TGTGCATCTC-CCAAAGTGTG-3) for TS. PCR cycling parameters were 3 min at 94°C, followed by 23 cycles of 30 s at 94°C, 30 s at 58°C, 45 s at 72°C, and a 7-min 72°C extension. Products were resolved on 1.5% agarose gels and stained with ethidium bromide. Quantitation of images captured using the ImageMASTER VDS gel documentation system (Amersham Pharmacia Biotech) was done with ImageQuant version 5.1 (Molecular Dynamics).

Statistical Analysis. Statistical significance within experiments was determined using Student's test (0.05). All experiments were repeated at least twice.

Results

Apoptosis of HeLa Cells is Unaffected by Oligodeoxynucleotide Treatment. In order to examine more closely the mechanism by which treatment with ODN 83 inhibits cell proliferation, the terminal deoxynucleotidyl transferase dUTP nick-end-labeling assay was used to determine whether apoptosis was induced in HeLa cells. Cisplatin treatment was included as a positive control and induced apoptosis in 85% of exposed cells after a 15 h treatment (Table 1). The low levels of apoptosis in HeLa cells were unchanged at 15 h after treatment with ODN 83, suggesting that the antiproliferative effects of the antisense ODN 83 might be due to a cytostatic, rather than an apoptotic, mechanism. Apoptosis levels were also not increased at 24 or 39 h after ODN 83 treatment (data not shown).

TS Antisense Oligodeoxynucleotide Induces a G_2/M Cell Cycle Block in HeLa Cells. Flow cytometric analysis was used to examine the cell cycle distribution of HeLa cells treated for various times with ODNs. There were substantial increases in the fraction of cells in G_2/M at 24, 36, and 48 h after treatment with the TS antisense ODN 83, compared

TABLE 1
Apoptosis levels in HeLa cells following treatment with ODNs
HeLa cells were treated with control scrambled ODN 32, TS antisense ODN 83, or cisplatin, and assayed for apoptosis after 15 h as described under *Materials and Methods*.

	ODN 32/50 nM	ODN 83/50 nM	Cisplatin 0	Cisplatin 40 μM
Experiment 1				
Proliferation rate[a]	100	16.77*		
Apoptosis[b]	6.98	7.13	0	85.44
Experiment 2				
Proliferation rate	100	8.56*		
Apoptosis	3.10	3.47		

* Significant difference from ODN 32-treated cells ($p < 0.05$, Student's t test).
[a] Proliferation rate relative to ODN 32-treated cells was determined in each experiment from cell numbers in control flasks, and calculated using the formula:
100 × [(final cell # + starting cell #) − 1] ÷ [(ODN 32 final cell # ÷ starting cell #) − 1]
[b] Percentage of apoptotic cells in each condition.

with the control scrambled ODN 32 (25–46 versus 18–22%, respectively) (Fig. 1). Increases in the fraction of cells in S phase at 24 and 36 h (19 and 12% in ODN 32-treated cells compared with 35% in ODN 83-treated cells) were essentially reversed by 48 h. Accumulation of cells in G_2/M was seen as early as 24 h, whereas the profile at 72 h closely resembled that of control cells. In contrast, treatment of HeLa cells with raltitrexed (1 μM for 2 h, followed by culture in drug-free medium for 48 h) resulted in cell cycle arrest exclusively in early S phase: 76% of raltitrexed-treated cells versus 22% of control cells were in S phase, whereas 23% of raltitrexed-treated cells were in G_0/G_1, versus 61% of control cells.

Effects of TS Antisense ODN 83 on HT29 Cells In Vitro. To determine whether the observed G_2/M arrest was p53-dependent, the effects of ODN 83 on a p53 mutant cell line, the HT29 human colon carcinoma, were examined. RT-PCR analysis showed that treatment of HT29 cells with the TS antisense ODN 83 reduced the levels of cytoplasmic TS mRNA but not GAPDH mRNA (Fig. 2A). Quantitation of the TS PCR product normalized to the GAPDH product from two independent RT-PCR experiments revealed 37% (1.53) and 43% (4.78) reductions in TS mRNA at 24 and 48 h, respectively, in cells treated with the TS antisense ODN 83 compared with cells treated with the control scrambled ODN 32. Flow cytometric analysis showed that HT29 cells, similar to HeLa cells, were blocked in G_2/M at 48 h after treatment with TS antisense ODN 83 (Fig. 2C). Quantitation of these data indicated that 43.0% of the ODN 83-treated cells were in the ç $_0$/G_1, 31.2% in S, and 25.8% in G_2/M phase, compared with 62.3, 25.2, and 12.5%, respectively, for ODN 32-treated cells. Again similar to HeLa cells, raltitrexed and 5-FU both induced G_1/S phase arrest in HT29 cells (Fig. 2, D and E). Treatment with TS antisense ODN 83 (100 nM) for 4 days inhibited proliferation of HT29 cells by 50.2% (S.E.M.   4.75,

4), compared with cells treated with the control scrambled ODN 32. Thus, antisense ODN-mediated down-regulation of TS mRNA and protein in HeLa and HT29 cells activates a novel G_2/M cell cycle block and inhibits cell proliferation in vitro.

Effect of TS Antisense ODN 83 on HT29 Tumor Growth In Vivo. Growth of human HT29 colon carcinoma cells in immunocompromised mice was examined to determine the antitumor efficacy of TS antisense as a single agent therapy. Compared with mice treated with the control scram-
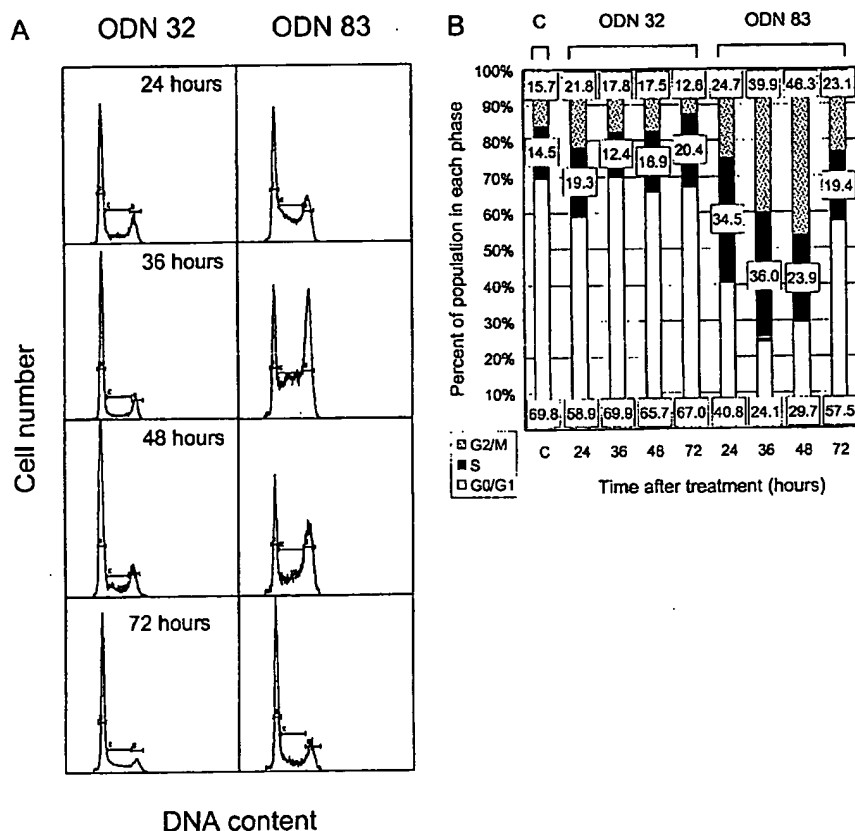
Fig. 1. Flow cytometric analysis of cell cycle distribution of ODN-treated HeLa cells. HeLa cells were collected at the indicated times after treatment with control scrambled ODN 32 (A, left) or TS antisense ODN 83 (A, right), stained with propidium iodide, and analyzed by flow cytometry. B, the percentage of cells in the G_0/G_1 (open region), S (filled region), and G_2/M (dotted region) cell cycle phases for control untreated HeLa cells (C), and for the histograms shown in A.

bled ODN 32, treatment with the TS antisense ODN 83 significantly inhibited tumor growth over the course of 4 weeks (Fig. 3). Growth of tumors in mice treated with the control scrambled ODN 32 was not significantly different from those injected with saline (0.217 to 0.982 for differences in tumor volume on days 7–27, Student's test, 5). Tumor growth delay was caused by the antisense ODN 83 at 7.5 or 11.25 mg/kg of body weight when the ODN was administered every 2 days commencing the day after tumor implantation. However, there was no effect at either concentration of ODN against tumors that had been allowed to grow untreated to a size of 400 mm³ prior to commencing treatment (Fig. 4). The ODNs were well tolerated, without significant delays in normal weight gain (0.37 to 0.76 for differences in mean body weight of mice treated for 28 days with saline, antisense ODN 83, or control ODN 32, Student's test, 10).

Distribution of Oligodeoxynucleotides in Vivo. We hypothesized that ODN delivery or penetration into larger tumors might be a limiting factor in this therapy. To examine this question, end-labeled ODN was used as a tracer for injection into mice bearing HT29 tumors. The radiolabel was evenly distributed in normal spleen, kidney, and liver, but it was relatively concentrated around the periphery of the tumor (Fig. 5A). There was a higher degree of cellularity around the periphery of the tumors (Fig. 5C) compared with the interiors (Fig. 5D), which were necrotic, as evidenced by cell shrinkage, increased extracellular space, and decreased hematoxylin-staining nuclei. The morphology of tumors from mice treated with the control and the antisense ODNs was

similar. There was no significant difference in the mitotic index between tumors in the two groups of animals.

Decreased TS Levels in HT29 Tumors in Nude Mice Treated with Antisense Oligodeoxynucleotide. To test whether in vivo administration of TS antisense ODN 83 down-regulates TS expression in HT29 tumors, a [6-³H]5-FdUMP binding assay was used to measure TS levels. Tumors were dissected from mice that had been treated with ODN every 2nd day for 28 days (the experiment shown in Fig. 3B) and the levels of TS were measured as described under *Materials and Methods*. Systemic administration of TS antisense ODN 83 caused a 43% decrease in TS protein levels within the tumors, compared with tumors from mice treated with the control scrambled ODN 32 (Table 2).

Discussion

We report that use of an antisense strategy to deplete human tumor cells of TS mRNA, rather than inhibiting the activity of existing TS enzyme with cytotoxic drugs, induced a distinctive response in cultured tumor cell lines. Antisense ODN treatment blocked cells at G_2/M without appreciable arrest at G_1/S and suppressed proliferation in the absence of a measurable increase in apoptosis. The reduced fraction of cells in G_0/G_1 at an early time following antisense ODN treatment (24 h) and the progressive increase in the fraction in G_2/M up to 48 h indicate that blockage at G_1/S followed by synchronous progression through the cell cycle cannot be invoked as a explanation. Between 48 and 72 h after antisense ODN 83 treatment, the proportion of cells in each cell
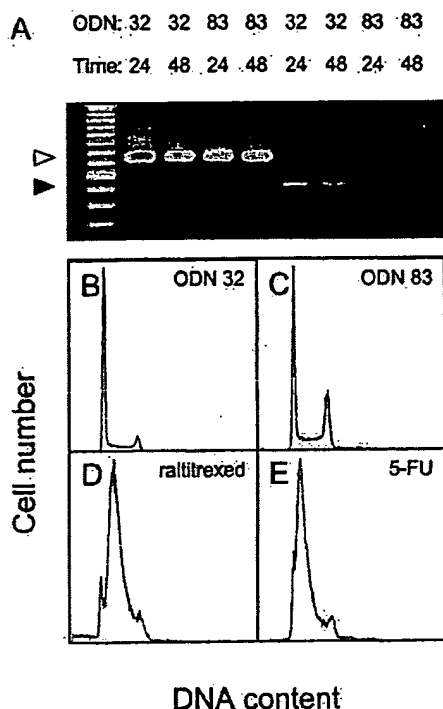
Fig. 2. TS mRNA levels and cell cycle profile in HT29 cells treated with ODNs in vitro. A, RT-PCR analysis of GAPDH (open arrowhead) and TS (filled arrowhead) mRNA levels in HT29 cells 24 and 48 h after treatment with control scrambled ODN 32 or TS antisense ODN 83. B–E, HT29 cells treated for 2 days with 200 nM control scrambled ODN 32 (C) or TS antisense ODN 83 (D), or HT29 cells treated for 4 h with 1 μM raltitrexed (E) or 0.1 mM 5-FU (F), then cultured for 2 days in drug-free medium. Cells were stained with propidium iodide and analyzed by flow cytometry.

Time (days after tumor implantation)

Fig. 3. Antitumor effect of systemic administration of TS antisense ODN 83. Nude mice were injected subcutaneously with HT29 tumor cells on day 0. On every 2nd day beginning on day 1, mice were injected intraperitoneally with 7.5 (A) or 11.25 (B) mg/kg of body weight of control scrambled ODN 32 (filled circles) or TS antisense ODN 83 (open circles), and tumors were measured every 4 days. Shown are the average tumor volume (± standard error) from 15 (A) or 5 (B) mice. *Significant differences ($p < 0.05$) from mice treated with the control scrambled ODN 32.

cycle phase returned to control levels. We previously reported that the proliferation rate of antisense ODN 83-treated cells returned to normal at that time (Ferguson et al., 1999). Thus, the antisense ODN-mediated decrease in TS mRNA and protein corresponds directly with G₂/M arrest and reduced pro liferation.

Several molecules are known to play critical roles in mediating arrest at the G₂/M boundary, particularly p53 (Agar wal et al., 1995; Stewart et al., 1995) and its downstream effector p21waf1 (El Deiry et al., 1993). The two human cell lines studied here, HeLa (cervical carcinoma) and HT29 (colon carcinoma), express widely disparate p53 levels. p53 is exceptionally low in HeLa cells, possibly related to unusual instability of the protein due to papillomavirus E6 protein expression (Hamada et al., 1996). Like many other human tumor cell lines, HT29 cells express high levels of mutant p53 (Rodrigues et al., 1990). The observation of a G₂/M block induced by antisense ODN treatment in cells with both high and low p53 expression suggests a p53-independent mechanism, reminiscent of the lack of dependence on p53 of thymineless death induced by direct inhibitors of TS protein (Munoz-Pinedo et al., 2001). The accumulation of cells in ç ₂/M, as opposed to G₁/S arrest induced by raltitrexed and 5-FU (Fig. 2) (Inaba and Mitsuhashi, 1994; Matsui et al., 1996), suggests that TS antisense treatment has consequences other than simply limiting the supply of thymidylate for DNA synthesis. Direct inhibitors of TS reduce enzyme activity by inactivating pre-existing TS protein, without di-
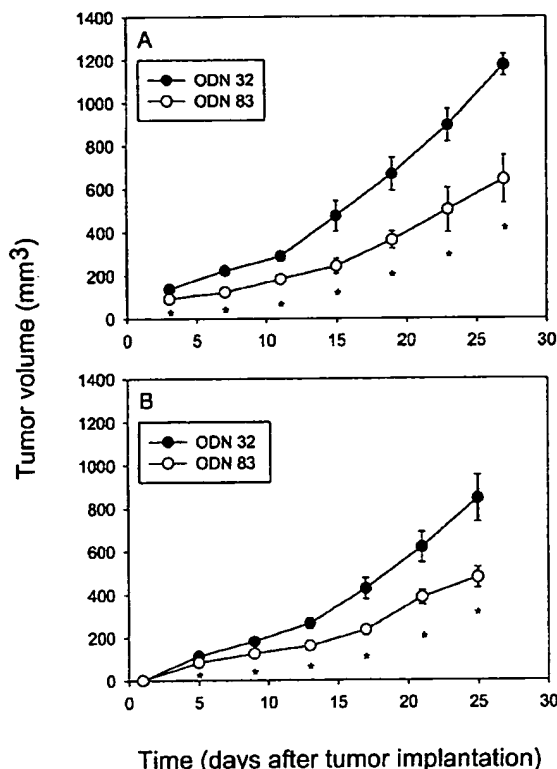
rectly influencing TS mRNA levels, and often result in increased TS mRNA translation. Antisense targeting, on the other hand, decreases both protein and mRNA levels. The association between decreased TS mRNA and protein levels and G₂/M cell cycle arrest observed in response to antisense targeting suggests that TS mRNA, TS protein, or both, mediate functions additional to catalysis of thymidylate production.

The complexity of control of TS expression provides multiple points where antisense treatment could interfere with additional functions to influence cell cycle and apoptotic pathways in novel ways. For example, TS mRNA levels and enzyme activity increase 10- to 20-fold as cells progress through cell cycle (Navalgund et al., 1980), while TS gene transcription rate is up-regulated only two to four times, suggesting that multiple post-transcriptional mechanisms play a major role in TS regulation (Johnson, 1994). TS protein has been reported to interact with its own and other mRNAs (p53 and c-myc), inhibiting mRNA translation and leading to decreased specific protein levels (Van Triest et al., 2000; Schmitz et al., 2001). In addition, antisense nucleic acids targeted to the TS mRNA translation start site stimulate TS gene transcription (DeMoor et al., 1998), possibly by interfering with associations between that region of TS mRNA and cellular components that function to suppress TS

Tumor volume (mm³)

- ● Saline control
- ○ 11.25 mg/kg ODN 32
- ▼ 11.25 mg/kg ODN 83
- ▽ 7.50 mg/kg ODN 83

2400 2000 1600 1200 800 400 0

0 5 10 15 20 25 30 35
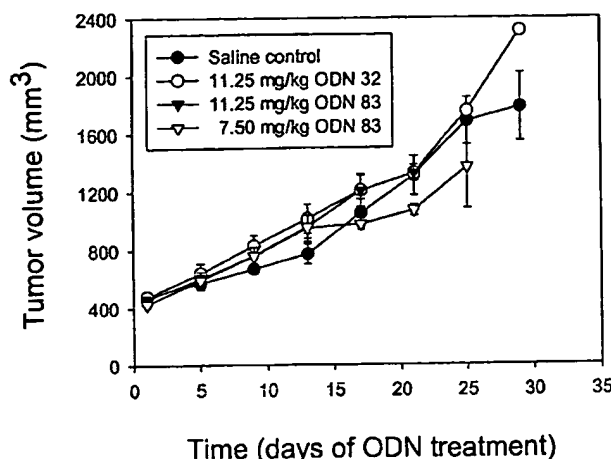
Time (days of ODN treatment)

Fig. 4. Systemic administration of TS antisense ODN 83 has no effect on larger, established HT29 tumors in nude mice. Nude mice were injected subcutaneously with HT29 tumor cells, and tumors were allowed to grow to a size of 400 mm³ before treatment with ODNs as described for Fig. 1. Shown are the average tumor volume (± standard error) from five mice treated with saline (filled circles), 11.25 mg/kg control scrambled ODN 32 (open circles), 11.25 (filled triangles) or 7.5 (open triangles) mg of TS antisense ODN 83 per kg of body weight.

gene transcription. Studies are under way in our laboratory to dissect the molecular signaling pathways that lead to cell cycle arrest at G_2/M following TS antisense ODN treatment.

In contrast to apoptosis induced by direct inhibitors of TS protein (Van Triest et al., 2000), we found no increase in cell death in response to cell cycle arrest by TS antisense ODN in vitro. Considering that imbalances in dTTP/dUTP levels and DNA damage caused by inhibitors of TS enzyme activity can result in induction of downstream events leading to apoptosis, this indicates that antisense targeting has novel consequences that do not simply achieve the same results as TS protein targeting by a different route. The potential of treatment with antisense reagents to induce G_2/M arrest and inhibit cell proliferation without enhancing cell death, and the mechanism by which this occurs, is a previously undescribed but important area of future investigation.

In the in vivo experiments described here, mice bearing HT29 tumors were treated every 2nd day with ODN, resulting in decreased TS protein levels and reduced tumor size (by approximately 50%) after 4 weeks of growth. No overt toxicity (i.e., weight loss) was apparent in animals treated with TS antisense ODN 83. Thus, antisense strategies to target TS have the potential to be effective cytostatic methods to reduce human tumor growth. Although there was no obvious change in mitotic index associated with reduced growth, only tumors greater than 600 mm³ in volume were assessed microscopically to determine the metaphase fraction. In fact, tumors allowed to grow to 400 mm³ prior to ODN administration were unaffected by antisense TS. Together, these data suggest that treatment with TS antisense ODN in vivo may ultimately be of greatest value in adjuvant therapy of residual or nascent disease rather than in treatment of gross tumors.

Typical of rapidly growing adenocarcinomas, the large, well developed HT29 tumors had necrotic centers and appeared to be poorly vascularized. This might result in poor ODN delivery to the interior of large tumors, consistent with imaging of radiolabeled ODNs that preferentially localized at the periphery of tumors. The tumor periphery also contained the greatest proportion of cells whose appearance was consistent with higher viability and proliferation rate. Although dissociation of radiolabeled phosphate from ODN is theoretically possible and a potential complicating factor in interpretation, persistence of proliferating cells in the presence of high antisense ODN levels suggests that the antisense reagent has diminished capacity to inhibit cell proliferation in larger tumors, or that decreased TS levels might have less influence on cell proliferation in macroscopic versus microscopic tumors. TS levels in tumor extracts were reduced by more than 40% by antisense TS treatment, and the possible presence of TS derived from mouse tissue or HT29 cells that failed to internalize ODN suggests that the observed reduction in TS protein underestimates the true effectiveness of antisense treatment. Although detailed examination of ODN distribution and effectiveness at the cellular level is required to resolve this issue, these data indicate a potential tumor size-dependent difference in response to TS antisense treatment.

The present results show clearly that systemic treatment with TS antisense ODN 83 as a single agent significantly delays HT29 tumor growth in nude mice (Fig. 3), similar to the effect of raltitrexed alone (data not shown). We demonstrated previously that HeLa cells treated in vitro with TS antisense ODN 83 are sensitized to cytotoxicity of 5-FUdR or raltitrexed (Ferguson et al., 1999), and further studies are under way to test the hypothesis that sensitization occurs in vivo in microscopic and/or macroscopic tumors. Such a combination treatment may be necessary to overcome drug resistance mediated by TS upregulation in tumors in response to anti-TS chemotherapy (Gorlick and Bertino, 1999). We have observed in vitro that antisense ODN 83 effectively sensitized drug-selected, highly resistant HeLa cells to 5-FUdR cytotoxicity (P. J. Ferguson, in preparation), supporting this hypothesis. Antisense down-regulation of several specific mRNA targets sensitizes cells to chemotherapeutic drugs in vitro. For example, antisense to protein kinase Cα enhances sensitivity to mitomycin C, vincristine, and 5-FU (Chakrabarty and Huang, 1996), and antisense to c-myb sensitizes cells to cisplatin (Del Bufalo et al., 1996). The use of antisense ODNs to down-regulate Bcl-2 (Miayake et al., 2000) or tubulin (Kavallaris et al., 1999; Kyu-Ho Han et al., 2000) enhances paclitaxel sensitivity in drug-resistant tumor cells. This rational approach to combat tumor cell chemotherapy resistance, also demonstrated for TS in vitro (Ferguson et al., 1999; P. J. Ferguson, in preparation), is an important ongoing area of investigation.

In summary, an antisense ODN targeting TS was shown to be an effective single-agent antitumor therapy in vivo. Distribution of ODNs into solid tumors may be a limiting factor in efficacy against larger tumors. Further in vivo studies to examine the potency of combination therapies using this antisense ODN in combination with raltitrexed and 5-FU are in progress. In HeLa and HT29 cells in vitro, down-regulation of TS mRNA and protein by treatment with TS antisense ODN 83 resulted in inhibition of proliferation, in the absence of increased cell death, via an immediate and sustained G_2/M cell cycle block. This was in contrast to the effect of TS-targeting chemotherapeutic drugs, which block cells in G_1/S and lead to increased apoptosis. Further studies are required to determine the molecular signaling pathways that mediate
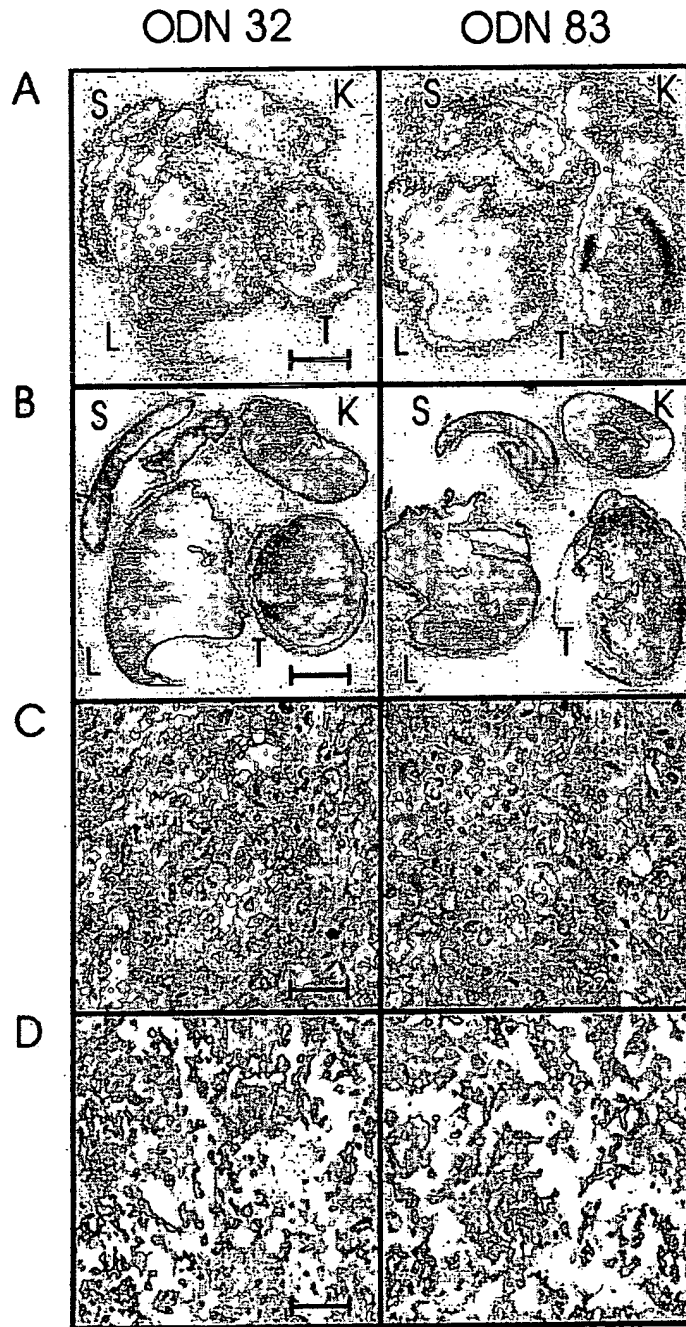
ODN 32   ODN 83

Fig. 5. Distribution of radiolabeled ODNs in HT29 tumors and normal mouse tissue. Mice bearing HT29 tumors that had been treated as described for Fig. 3B with control scrambled ODN 32 (left panels) or TS antisense ODN 83 (right panels) were injected with a trace amount of end-labeled ODN. A, radioactive decay images from sections of mouse spleen (S), kidney (K), liver (L), and HT29 tumors (T). B, hematoxylin and eosin staining of adjacent sections. Photomicrographs of the periphery (C) and the interior (D) of the tumors show cellular morphology. Scale bars, 5 mm (A and B) and 40 μm (C and D).

TABLE 2

TS protein levels in HT29 tumors in mice treated with ODNs

Tumor samples from mice treated with control scrambled ODN 32 or TS antisense ODN 83 were collected and analyzed for [6-³H]5-FdUMP binding as described under *Materials and Methods*. Data shown are for tumors described in Fig. 3B.

Treatment	TS Levels[a]	% Reduction[b]
ODN 32	73.8 ± 8.6	
ODN 83	42.2 ± 13.9*	42.8

* Significant difference from ODN 32-treated mice (p = 0.003, Student's t test).
[a] [6-³H]5-FdUMP binding in tumor extracts, expressed as nmol/mg of protein (± S.E.M., n = 5).
[b] Reduction in TS level due to ODN 83 treatment, calculated as percentage of that in tumors from ODN 32-treated mice.

the G₂/M arrest and to ascertain how this phenomenon might be further exploited in novel antitumor therapies.

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Address correspondence to: Dr. J. Koropatnick, Cancer Research Laboratories, The London Regional Cancer Center, 790 Commissioners Rd., London, ON, Canada, N6A 4L6. E-mail: jkoropat@uwo.ca